BASES OF THE NUCLEIC ACID OF FOWLPOX VIRUS AND HOST DEOXYRIBONUCLEIC ACID

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ABSTRACT

RANDALL, CHARLES C. (University of Mississippi School of Medicine, Jackson), LANELLE G. GAFFORD, AND ROBERT W. DARLINGTON. Bases of the nucleic acid of fowlpox virus and host deoxyribonucleic acid. J. Bacteriol. **83**:1037–1041. 1962.—The nucleic acid of fowlpox virus obtained from purified inclusions was identified as deoxyribonucleic acid (DNA). The base ratios determined on whole virus and on isolated DNA were practically identical. The molar ratios of purines to pyrimidines were approximately unity. The ratio of adenine plus thymine to guanine plus cytosine was 1.8.

The base ratios of DNA obtained from chick red cells were approximately the same as other animal DNA preparations reported in the literature. It is of interest that the molar proportions of host DNA are significantly different from viral DNA.

Previous preliminary reports (Randall and Gafford, 1960, 1961) indicated that the inclusion bodies so characteristic of fowlpox infection can be obtained in quantity, largely free of contaminating cell debris, and are a rich source of viral particles, the nucleic acid of which is predominantly deoxyribonucleic acid (DNA). It has been demonstrated (Randall and Gafford, 1962) that the inclusion is not a simple structure, but is composed of viral particles suspended in a complex matrix of abundant lipid and protein composition with definite organization as visualized by the electron microscope (Randall, Gafford, and Arhelger, 1961).

This report concerns an investigation of the nucleic acid of viral particles isolated from the typical inclusions of the disease produced in the chick scalp. The base analyses are compared with host DNA. The results show that the purified virus contains only DNA, and the quantitative purine and pyrimidine composition has been shown to be different from that of host DNA.

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MATERIALS AND METHODS

Virus. The strain of fowlpox virus (FP) and method of inoculation of 1-day-old cockerels have been described (Randall et al., 1960).

Isolation of viral inclusions. The details of the methodology of isolation and purification of the inclusions have been published (Randall and Gafford, 1962). Chick scalps were excised 7 days after inoculation and were stored overnight in 1% trypsin in Sorenson's phosphate buffer (pH 7.6). Briefly, the superficial epithelium and the hyperplastic feather follicles could then be separated from the connective tissue. Since the above process softened the epithelial tissues to a marked degree, the trypsin solution and saline wash contained numerous free inclusions, which were recovered by centrifugation. The sediment and the epithelial structures were suspended in fresh trypsin with glass beads and were stored at 4 C for 18 to 20 hr, occasionally agitated by hand, effectively freeing most of the inclusions. Purification was accomplished by 10 to 15 cycles of centrifugation and washing with phosphate-buffered saline (PBS) until the supernatant fluid was clear and microscopic examination showed little or no debris.

Isolation and purification of virus. Isolation of the virus from inclusions offers the distinct advantage of starting with material that contains little contaminating cell debris (Randall and Gafford, 1962). Suspensions of purified inclusions in PBS were taken as the starting material. A typical sample, containing about 1.2×10^8 inclusions, was centrifuged (clinical International model 2) at $535 \times g$ for 15 min, and the supernatant fluid discarded. The sediment was resuspended in distilled water without difficulty,

and the process repeated for several cycles. The inclusions at this point were estimated to be associated with less than 1% debris. After the final washing, the inclusions were resuspended in 25 to 30 ml of distilled water and treated for 15 min in a Raytheon 9 KC oscillator. Longer periods of sonic treatment were tried, but did not seem to result in an appreciable increase in the yield of virus and definitely decreased the viability of the particles. The suspension was then centrifuged at 667 × *g* for 15 min to sediment fragments of inclusions and larger debris. Some virus is sedimented at this speed, and the yield was increased by washing the sediment with distilled water, recentrifuging, and combining the supernatant fluids, which are then centrifuged at 27,000 × *g* for 20 min in a refrigerated Servall RC-2 centrifuge. The homogeneous pellet was resuspended in 5 to 6 ml of distilled water, and subjected to sonic vibration for 1 to 2 min to assure maximal separation of the viral particles. The resulting suspension was then centrifuged in a refrigerated Spinco model L ultracentrifuge at 14,830 × *g* for 20 min. The supernatant fluids from the two high-speed centrifugations contained the major portion of the inclusion matrix material, the composition of which is currently under investigation. The virus button was examined for purity by electron microscopy, and if necessary the cycle of centrifugation was repeated. The final product was resuspended as before in a known volume for subsequent examination.

Electron microscopy. Various dilutions of the purified elementary bodies in distilled water were introduced into the cells of a SU particle counting rotor (Ivan Sorvall, Inc.); on the bottom of each cell was a layer of 2% agar. Operation of the RC-2 centrifuge for 15 min at 5,020 × *g* was sufficient to deposit all the particles on the agar. The supernatant fluid was decanted and the agar carefully removed to a petri dish, on the inside cover of which had been deposited several drops of 2% osmic acid. After 5 min of fixation, the agar was removed and a collodion pseudoreplica made from its surface. This was chromium shadowcast at a grazing angle of 11° and photographed in an RCA EMU-3 electron microscope (50 kv accelerating potential), to obtain an estimate of the purity of the preparations. This procedure is essentially that of Sharp (1958). The preparations showed very little or no visible impurities.

Nucleic acid standards. Highly polymerized calf thymus DNA standard was obtained from Mann Research Laboratories, Inc., and the standard bases from California Corporation for Biochemical Research.

Preparations of normal host tissue. The epithelium from 7-day-old chicks (to correspond with the infected animal of the same age) was removed by trypsinization as described by Randall and Gafford (1962). Red cells (RBC) were obtained from normal and infected chicks of the same age and washed repeatedly in 0.15 M NaCl, and the nuclei were isolated in 0.01 M citric acid by the method of Dounce (1943). The nuclei were estimated to be contaminated with 5 to 10% RBC. The preparation, however, was quite satisfactory for the isolation and subsequent chromatography of DNA, as the interfering pigment was largely eliminated.

Preparation of nucleic acid. The viral preparations (averaging 10 to 15 mg per sample), RBC nuclei, and epithelium were extracted twice with 0.4 N perchloric acid (PCA) at 4 C to remove the acid-soluble fraction. The residue was washed repeatedly with cold 95% ethanol and exhaustively extracted with chloroform methanol (2:1). After lipid extraction, the material was washed three times with cold 95% ethanol and dried under N_2.

Alkaline hydrolysis. The residues from above, with the exception of all the whole-virus preparations, were treated with 1 N KOH at 37 C (overnight) according to Davidson and Smellie (1952) with minor modifications. The alkali digest was chilled to 4 C, and the DNA and protein precipitated by the addition of 70% PCA (to pH 1). The supernatant fluid should contain the ribonucleic acid (RNA) fraction (as mononucleotides) if present. The precipitate was washed with cold PCA and cold distilled water, transferred quantitatively to 3-ml conical centrifuge tubes, dried under N_2, and stored under vacuum in a desiccator in the cold until ready for use.

Acid hydrolysis and chromatography. The various samples of DNA, including the standard and preparations of whole virus, were hydrolyzed with 70% PCA at 100 C for 1 hr. The resulting bases were chromatographed and quantitated essentially as described by Bendich (1957). Hydroly-

zates and standard mixtures of bases were spotted in 10- to 25-μliter amounts on Whatman no. 1 paper, chromatographed at controlled temperature (25 C) in isopropanol-HCl solvent, and allowed to run for 24 hr, or until the front movement was 40 to 45 cm from the point of origin. The characteristic spots were located under ultraviolet light (UV), excised, and eluted in 10 ml of 0.1 N HCl. The eluted bases were read in a Perkin-Elmer model 4000 automatic spectrophotometer using calibrated 10-cm long path cells, and the instrument-drawn curves were determined over a UV-wavelength range from 320 to 220 mμ. The spectra were compared with standard solutions of the bases, chromatographed, and eluted under identical conditions, to establish the identity and purity of the compounds. Agreement with data published by Bendich (1957) was excellent. Molar base ratios were determined by the differential extinction technique and expressed as percentages of total bases recovered. Recovery of the standard purine-pyrimidine mixtures was within $\pm 3\%$, except for thymine which averaged minus 5% recovery.

It is pertinent to comment on the attempted analysis of chick epithelial DNA. Unfortunately, normal epithelium does not appear to be good material for chromatography in our hands. The base spots were easily visible and well isolated, but there was a visible streak under the UV that ran the entire length of the paper. This streak was present in the normal epithelium lanes on both washed and unwashed paper, but was not present on the same papers in the blank lane or the lanes containing the standard DNA. Maximal absorbance of eluted samlpes was at 254 mμ with significant absorbance at the absorption maximum of each of the bases. Therefore, use of this material was abandoned.

Phosphorus. Phosphorus was determined by the method of Soule, Marinetti, and Morgan (1959).

Enzymes and treatment of whole virus. Crystalline beef pancreatic deoxyribonuclease and ribonuclease were obtained from Mann Research Laboratories, Inc. The enzyme buffer solution consisted of 0.11 M NaCl, 0.01 M Na_2HPO_4, and 0.005 M $MgSO_4$ dissolved in distilled water.

A sedimented sample of purified virus was suspended in 4 ml of buffer, and was subjected to sonic treatment for 1 min to disperse the particles thoroughly. An equal volume of the enzyme solution was added (final concentration of each nuclease was 10 μg/ml), the sample was incubated for 1 hr at 37 C, chilled in an ice bath, and centrifuged at 14,830 $\times$ *g* for 20 min in a Spinco model L ultracentrifuge. The supernatant fluid was discarded; the sediment was resuspended in distilled water and again subjected to sonic treatment for 1 min. A portion was removed at this point for examination with the electron microscope, as described earlier. The preparations revealed the presence of what appeared to be intact viral particles. No counts or attempts at quantitation were made. The remainder of the sample was treated in the described manner for chromatography.

RESULTS

Test for RNA by chromatography. It was necessary to establish whether the preparations of whole virus contained RNA, either as an integral part of the particle or as a contaminant. Chromatograms of whole virus, hydrolyzed with $HClO_4$, were examined for possible uracil. The ratio of uracil to thymine would be expected to give an approximate proportion of RNA to DNA. Areas colorless to UV but corresponding to the location of uracil were removed from the chromatograms. In no case did the absorption curve exhibit the 260-mμ peak of uracil, so the very small peak (274 mμ) was most likely owing to other degradative compounds of viral hydrolysis. With the method of differential extinction, no uracil was detected. It is important to inquire as to what the upper limit of RNA (uracil) might be, as determined by the sensitivity of the technique. As little as 2 μg of uracil per spot could be detected by UV. Analysis of the spectrophotometric data indicates that if the virus contains any RNA the relative amount would be less than 0.5%. Chemical analysis of purified virus by the orcinol technique shows no detectable ribose.

DNA composition. Chromatograms of the various preparations of FP, either whole virus or viral DNA, showed only the four main constitutive bases (Table 1). No attempt was made to separate methylcytosine from the cytosine; therefore, this base, if present in small quantities, would have gone unrecognized. Any 5-hydroxymethylcytosine would have been destroyed during hydrolysis with $HClO_4$ (Wyatt and Cohen, 1953).

TABLE 1. *Base composition of whole virus and DNA isolated from virus and host red cells (with other DNA's for comparison)*

Material analyzed	Number of analyses	Mean estimated content of bases (moles/100 moles)				Ratio of base pairs
		Adenine	Thymine	Guanine	Cytosine	
Viral DNA	4	32.3	32.6	18.0	17.2	1.84
Fowlpox virus, whole	3	32.1	33.3	17.9	16.7	1.89
Fowlpox virus, whole, nuclease treated	5	31.6	33.3	19.5	15.7	1.84
Normal chick red cell DNA	5	27.9	29.4	21.2	21.5	1.34
Infected chick red cell DNA	10	27.0	29.7	21.6	21.8	1.31
Calf thymus standard DNA	10	28.4	27.5	23.1	21.1	1.26
Chicken red cell DNA[a]		28.4	28.8	21.3	21.6	1.33
Chicken spleen DNA[b]		29.5	27.7	22.4	20.4	1.34
Calf thymus DNA[c]		28.2	27.8	21.5	22.5[d]	1.27

[a] Chargaff (1955). (Arbitrary average of two separate analyses.)
[b] Kirby (1959).
[c] Wyatt (1951).
[d] Includes 1.3% methylcytosine.

Within the limitations of the method, the amount of any exotic base, if present, must be too small to be detected.

It is apparent that, regardless of the material analyzed, the viral DNA bases are paired, adenine equal to thymine, and guanine approximating cytosine, as would be expected. The molar dissymmetry ratio is surprisingly high (1.8), comparable to certain bacterial viruses; the same ratio for vaccinia virus is 1.46 (Wyatt and Cohen, 1953).

We have taken the precaution to determine exhaustively the base ratios of chick and calf thymus DNA, as a control of our methods, rather than to depend exclusively on data quoted from others. The averages were compared with values from the literature, as shown in Table 1, and were found to be in reasonable agreement.

Phosphorus content. As a check on the relative purity of the several preparations, the phosphorus content was determined on some random samples of the materials shown in Table 2. Duplicate samples of the PCA hydrolyzate were taken for chromatography and for phosphorus analysis. The DNA content of the samples was estimated from the total recovery of the four bases. Virtually all of the phosphorus in the PCA hydrolyzate can be accounted for by the recovered bases. Therefore, no appreciable amounts of bases were undetected by the chromatographic methods, nor was there any significant contamination of the hydrolyzate with non-DNA phosphorus-containing compounds. The high recovery from the nuclease-treated, exhaustively extracted, whole virus preparations is further proof of the absence of any RNA in this virus. The analysis of viral DNA was not accomplished because of the demands of chromatography.

TABLE 2. *Mean phosphorus content of fowlpox virus compared with chick and calf thymus DNA*

Material	Number of preparations	Number of analyses	Recovery (sum of bases/phosphorus)	DNA phosphorus content
				%
Chick RBC DNA	2	2	0.92	9.7
Nuclease-treated fowlpox virus, whole	2	2	0.98	9.1
Calf thymus DNA	2	2	1.05	8.5[a]

[a] Vendor's analysis: 8.6% P.

DISCUSSION

The work presented in this paper shows that FP is a "DNA" virus and does not contain any demonstrable RNA. The fact that only one type of nucleic acid was identified is consistent with data for other viruses (Shäfer, 1959). If comparison is restricted to mammalian viruses containing DNA, studies of the base or nucleotide composition are limited to vaccinia virus (Wyatt and Cohen, 1953) and polyoma virus (Smith et al., 1960). Both vaccinia and FP are members of the pox group and invite pertinent comparison. Both

viruses are of the adenine-thymine type, and in both cases, as would be anticipated, the molar ratios of purine to pyrimidine are close to unity. The pairing of the bases presumably indicates that the DNA of FP (and vaccinia) is double stranded.

The base ratio (1.8) is significantly different from the values for vaccinia virus. It is worth emphasizing that the base ratios of the several types of FP material are significantly different from chick erythrocyte DNA. This difference lends support to the claim that there is no significant contamination with host DNA.

These differences in DNA invite some comment. It is well known that fowlpox is a disease in which hyperplasia of the ectoderm is conspicuous with concomitant formation of intracytoplasmic inclusions. One may well speculate about the DNA of the cell which has been induced to multiply as the result of the infection. The DNA of the nucleus of the hyperplastic cell may or may not be the same as normal chick DNA. It is possible that a different DNA is synthesized in two apparently separate areas of the cell, one in the nucleus and the other in the virus. This system appears to be an excellent model for investigation of the mechanism of hyperplasia and virus synthesis.

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